Annals of Clinical and Analytical Medicine
Original Research

Detection of Human Papillomavirus using polymerase chain reaction methods in transitional urothelial bladder cancer

Human Papillomavirus and urothelial bladder carcinoma

Mehmet Yıldızhan [1] , İzzet Koçak[2], Sevin Kırdar[3], Nil Çulhacı[4]
[1] Department of Urology, Ankara City Hospital, Ankara
[2] Department of Urology, Adnan Menderes University, Medicine Faculty, Aydin
[3] Department of Microbiology, Adnan Menderes University, Medicine Faculty, Aydin
[4] Department of Pathology, Adnan Menderes University, Medicine Faculty, Aydin, Turkey

Abstract
Aim: In this study, we aimed to evaluate the role of low- and high-risk human papillomavirus (HPV) types in the etiology of different stages of transitional urothelial bladder carcinoma (TUBC).
Materials and Methods: The medical records of 212 patients operated between 2009 and 2014 were analyzed retrospectively. One hundred thirteen patients with TUBC who underwent transurethral resection and partial/total cystectomy with a diagnosis of bladder cancer were reviewed and compared with 99 control patients with non-neoplastic disorders. Formalin-fixed and paraffin-embedded archival tissue samples were used for deoxyribonucleic acid (DNA) extraction. Specimens were analyzed using polymerase chain reaction (PCR) with HPV-specific general primers set for the detection of viral DNA. PCR-positive samples were also tested using HPV type-specific primers with the same method. The presence of HPV was evaluated using MolecuTech REBA HPV-ID.
Results: The median ages of the patients in the cancer and control groups were 68.5 (24-89) and 63.5 (28-83), respectively. None of the control patients exhibited HPV-DNA positivity. However, HPV-DNA positivity was observed in four patients (3.5%) in the cancer group. HPV types 6 and 84 are regarded as low risk, and 53 and 66 as high risk. There was no correlation between HPV infection and tumor development (x2 df(1)=3.572, p=0.125). No statistically significant relationship was observed between HPV-DNA positivity and tumor grade. However, a statistically significant association was determined between smoking and the development of bladder cancer (p<0.001).
Discussion: Our study findings indicate a low prevalence of HPV infection in FFPE bladder cancers, and suggest that HPV infections are unlikely to play a major role in the development of TUBC.

Keywords
Bladder cancer; Human papillomavirus; PCR; Transitional cancers

DOI: 10.4328/ACAM.20284 Received: 2020-07-20 Accepted: 2020-08-17 Published Online: 2020-09-07 Printed: 2021-04-01 Ann Clin Anal Med 2021;12(4):353-357
Corresponding Author: Mehmet Yıldızhan, Ankara City Hospital, Department of Urology, 06600-Bilkent, Ankara, Turkey.
E-mail: dr.mehmetyildizhan@gmail.com GSM: +90 5335499373 P: +90 3125526000 / 621549
Corresponding Author ORCID ID: https://orcid.org/0000-0001-8592-0874

Introduction

Bladder cancer is the seventh most common cancer among men worldwide, and the 11th most common cancer in both genders [1]. The incidence of bladder cancer and mortality rates vary from country to country due to differences in risk factors, detection and diagnostic practices, and the availability of treatments [2]. Several potential risk factors have been identified, including tobacco smoking, occupational exposure to aromatic amines, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons, exposure to ionizing radiation, bladder schistosomiasis, chronic urinary tract infection, urinary calculi, and chronic irritation or inflammation of the urothelium [2]. The World Health Organization estimates that approximately 12% of cancer cases worldwide are caused by viral infections [5]. Various bacterial or viral agents in the bladder have been shown to cause dysplastic changes in the bladder epithelium and ultimately to lead to cancer [4].

Human papillomavirus is the principal agent of sexually transmitted diseases, and can cause cancers in different anatomical regions at differing prevalences [5]. The potential role of high-risk human papillomavirus (hrHPV) in the development of cancer of the cervix, penis, vulva, anus and oropharynx is already well established in a publication of the International Agency for Research on Cancer in 2007. The presence of hrHPV is the main initiator factor for dysplasia, and eventually cancer [6]. Since the urinary bladder is in direct connection with the genital area through the urethra, HPV may also play a causative role in bladder cancer. HPV is associated with the development of a variety of proliferative lesions, including papilloma, intraepithelial neoplasia, and invasive cancer. Since HPV is the leading infectious cause of human cancers, this makes bladder HPV infection of particular medical importance. Advances in HPV diagnosis may have immediate implications for the fight against cancer [6].

Material and Methods

The research was performed as a retrospective, cross-sectional, descriptive study. The medical records and bladder tissue specimens of the 212 patients operated in our clinic between 2009 and 2014 were analyzed retrospectively. The cancer group consisted of 113 patients who underwent transurethral resection and partial/total cystectomy with a diagnosis of bladder cancer, the pathology being confirmed as TUBC. The control group consisted of 99 patients with benign bladder pathologies. Patients without TUBC pathology were excluded from the study. The control group consisted of patients who underwent transurethral resection for suspected mucosal appearance and/or luminal mass with no history of bladder cancer, and whose pathology was reported as benign [inflammation, inverted papilloma, papilloma, and cystitis).

Clinical information such as pathology reports, and data for age, education, smoking status, sexuality, age of sexual debut, marital status, and present history of sexually transmitted infections were collected from the hospital database and patient charts. Patients were categorized as smokers (currently active smokers or with a history of smoking) or non-smokers (never smoked) for a common definition.

Formalin-fixed and paraffin-embedded (FFPE) archival tissue samples were used for deoxyribonucleic acid (DNA) extraction. Tissue samples 4 micrometers (μm) in thickness obtained from paraffin blocks after fixation with 10% neutral buffered formalin solution and hematoxylin-eosin (HE) staining were reviewed again. Specimens were analyzed using polymerase chain reaction (PCR) with HPV-specific general primer set for the detection of viral DNA. PCR-positive samples were also tested with HPV type-specific primers using the same method. Presence of HPV was analyzed using MolecuTech REBA HPV-ID. Written informed consent was obtained from the patients who participated in this study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Adnan Menderes University research with ADU-BAP-TPF 2014/55 permit number and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards, with ''Regulations on Pharmaceutical Research", enforced by the Ministry of Health of Turkey published in the 27089 numbered Official Journal dated 23 December 2008 and also with other regulations published at a later date.

MolecuTech REBA HPV-ID test

This test can identify 18 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69 and 73) together with the possible high-risk type 34, and 13 low-risk group HPV types (6,11, 32, 40, 42,43, 44, 54, 70, 72,81, 84, and 87). The target of the test is the L1 gene, which is expressed in the late stages of infection. The test includes nested PCR and reverse hybridization steps. A commercial NucleoSpin Tissue mini-column (Macherey-Nagel, Duren, Germany) isolation kit was used for paraffin removal and deoxyribonucleic acid (DNA) isolation from the tissue. DNA isolation was performed according to the manufacturer's instructions. PCR amplification of HPV-DNA was performed following the manufacturer's instructions.

Evaluation

The strips were glued to the evaluation table, and the control line and the expected bands were matched. In the evaluation of each band

- The marker line indicates the top line of the membrane (Figure),
- The hybridization control (HC) line will appear if the chromogenic reaction is correctly prepared (Figure),
- The HPV universal probe band will form if no genotype of HPV is found (Figure) and,
- Each HPV type was assessed by observing the presence of band lines at the location (Figure).

Statistical Analyses

SPSS 22.0 for Windows software (IBM, Illinois, USA) was used for statistical analyses. Descriptive statistics were employed for assessing normality of distribution. Descriptive data were expressed as median values (min-max). The Chi-square and Fisher's exact tests were used to evaluate consistency between categorical variables. The statistical threshold value was set at $p < 0.05$.

Table 1. Distribution of cancer cases by pathological stages

Pathological stage	N	%
Low malignant papillary urothelial carcinoma (LMPUN)	15	13.3
pTa urothelial carcinoma	65	57.5
PT1 urothelial carcinoma	17	15
PT2 urothelial carcinoma	16	14.2

Table 2. Characteristics of patients with Human Papillomavirus (HPV) positivity

No	HPV type	HPV risk group	Age	Gender	Smoking status	Cancer grade	Pathological stage
1	Type 6	Low risk	63	Male	Positive	Low grade	pT1
2	Type 53	High risk	71	Male	Positive	Low grade	pT1
3	Type 66	High risk	88	Male	Positive	Low grade	pT1
4	Type 84	Low risk	74	Male	Positive	High grade	pT1

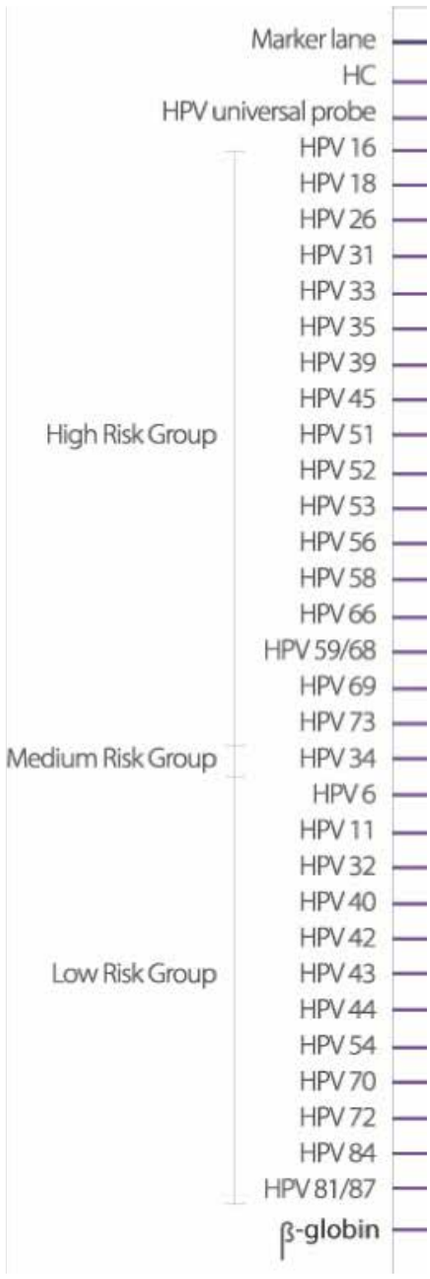

Figure. Human Papillomavirus type identification chart

Results

The median ages of the patients in the cancer and control groups were 68.5 (min: 24, max: 89) and 63.5 (min: 28, max: 83) years, respectively. Ninety-two (81.4%) of the patients in the cancer group were men, compared to 73 (73.7%) in the control group. Smoking rates were statistically significantly higher in the cancer group compared to the control group (p<0.001).

In terms of pathological cancer stages, 15 (13.3%) patients had papillary urothelial neoplasm of low malignant potential (PUNLMP), 65 (57.5%) had Ta urothelial carcinoma, 17 (15%) had T1 urothelial carcinoma, and 16 (14.2%) had T2 urothelial carcinoma. One patient with pT1 had comorbid carcinoma in situ, and two patients with pT2 exhibited squamous differentiation (Table 1). Low grade differentiation was seen in 64.6% (73) of the cancer group, and high grade differentiation in 35.4% (40). Pathological results of the patients in the control group were inflammation (acute and / or chronic) in 74 patients, polypoid cystitis in 14, inverted papilloma in two, papilloma in two, and cystitis cystica or von Brunn's islands in eight.

Genital warts or sexually transmitted diseases were positive in nine (9.1%) and 12 (10.6%) patients in the cancer and control groups, respectively. The difference was not significant (p=0.710). HPV positivity (type 53) was detected in only one of the 21 patients with a positive history.

HPV positivity was detected in four patients (3.5%) using the multiplex PCR method. The HPV types involved were 6, 53, 66 and 84. HPV types 6 and 84 are in the low-risk group, and types 53 and 66 are in the high-risk group. The cancer was in the pathological T1 stage in all HPV-positive patients. HPV types 6, 53 and 66 cause high-grade urothelial cancer, while HPV type 84 causes low-grade cancer (Table 2).

All the HPV-positive patients were men, and smokers. None of the four patients had a history of sexually transmitted disease or treatment. No condyloma-like lesions were observed in the genital or perineal regions. HPV positivity was not detected in any of the women in the cancer group. There was no correlation between HPV infection and tumor development (x2 df(1)=3.572, p=0.125). However, a statistically significant association was observed between smoking and the development of bladder cancer (p<0,001).

Discussion

According to U.S. data, bladder cancer is more than 2.5 times more common in men than in women [1]. In the present study, bladder cancer was four times more common in men. Bladder cancer is largely a disease of the elderly. The median age of our patients was 68.5 (min: 24, max: 89) in the cancer group and 63.5 (min: 28, max: 83) in the control group. Our findings confirm that bladder cancer is more common in the elderly population.

The relationship between HPV and TUBC is thought to be caused by the tendency of the virus to involve the epithelium and the anatomical proximity of the urethra to the bladder. This suggests that the male urethra may have a reservoir function and thus be implicated in at least the initial stage of bladder cancer. A carcinogenic role of hrHPV has been demonstrated in many previous studies [7-9]. And, also it was seen higher in male population according to our results.

The first study examining the relationship between HPV and bladder cancer was performed using the immunohistochemical (IHC) method with biopsy specimens in 1988 [9]. In 1992 Anwar and Phil showed that PCR was highly sensitive compared to IHC methods [10]. Several studies have investigated the relationship between bladder cancer and HPV using deparaffinized tissues with PCR methods [11-13]. The frequency of HPV as an etiological agent in studies evaluating the etiological role of HPV in TUBC pathogenesis varies between 0% and 80% [12-14]. This wide discrepancy in HPV detection rates between studies is due to problems in tissue fixation and DNA preparation and amplification, as well as problems that arise during sampling, contamination, the sensitivity of the scanning systems involved, and geographical differences [15,16].

There is still no consensus concerning the best molecular method for obtaining HPV-DNA in FFPE tissues. However, PCR with type-specific primers maintains remains the gold standard method by ensuring an overall HPV-DNA detection rate of 33.2% positivity in bladder cancer [17]. The commercial kit used in the present study can detect a total of 65 HPV types in the low-, medium-, and high-risk groups. However, technical failures in the methods employed to obtain DNA from deparaffinized tissue may explain the low rate of positivity. The principal difficulty in using FFPE tissues in genomic studies lies in the low quantity and quality of nucleic acid obtained from the blocks. The purpose of the FFPE method is to ensure that cellular proteins are preserved for future IHC-based studies, rather than providing good protection of nucleic acids. Fragmentation and degradation of nucleic acids due to chemical modification, and cross-linking to proteins and nucleic acids via amino groups (methylene bridges) can be caused by the formalin fixation process [18-21]. The extent of the potential damage to DNA resulting from the formalin fixation process is not entirely clear, although several different DNA damage mechanisms have been proposed. Formalin oxidized to formic acid causes breaks in nucleic acid in FFPE tissue. In order to obtain high-quality DNA, the tissue must be fixed with 10% buffered formalin for one day. The DNA quality of tissues treated for two days is 50% lower than that of one-day treatment. Keeping FFPE blocks in unsuitable environments, such as hot and humid conditions, and a long storage time can accelerate this process. At the same time, small and thin tissue to be fixed with formalin exhibited greater penetration by formalin and showed that DNA damage may increase. For all these reasons, the use of the DNA sample obtained for genomic analysis entails a number of limitations [22]. Studies have previously reported different success rates in processing FFPE samples for DNA sequencing [23,24]. This wide variability in sample viability will be affected by the age of the tissue block, the quality of DNA input, and the DNA sequencing methodology.

We used FFPE blocks obtained from the pathology archive for patients operated over a five-year period. A prolonged waiting period may cause tissue damage in FPFE blocks. Technical difficulties in block storage and deparaffinization may have caused our low HPV-DNA recovery rates. The number of samples in the control group was lower than that in the cancer group. This once again suggests the presence of formalin-induced DNA damage in low-volume samples. Although HPV has also been detected in benign bladder pathologies, it was not observed in our control group. Re-examination of the pathology reports of four patients in the cancer group were re-examined and revealed that at least 5 cc of tissue sample had been removed in all four cases and sent to the pathology lab.

Common virus types, such as HPV types 16 and 18, which have been found to be associated with malignancy in previous studies [15] were not observed in the present research. Detection of types 6 and 84 in the low-risk group with low potential for cancer, and of types 53 and 66 in the high-risk group, does not support the view that hrHPV causes cancer at a higher rate. This is because two types of viruses implicated in the etiology of benign lesions such as genital warts were found to be associated with bladder cancer in the present study. Low-grade cancer was detected in three out of four patients with HPV positivity, while high-grade cancer was detected in only one patient. The presence of HPV type 84, from the low-risk group, in a patient with high-grade cancer does not support the idea that HPV causes higher risk cancers. The presence of a history of smoking in all four patients with HPV positivity suggests a more significant etiological relationship with smoking. The present study observed a statistically significant difference between smoking and cancer detection compared to the control group ($p < 0.001$).

HPV-DNA positivity being observed in four patients suggested that HPV should not be implicated as an etiological agent in the development of transitional bladder cancer. HPV types 16 and 18, which were the most common types in other studies, were not detected in any of our patients. The relationship between the stage and grade of the tumor and the presence of HPV could not be evaluated due to the lack of sufficient HPV positivity. However, a statistically significant relationship between smoking and bladder cancer was shown in our research, in agreement with other studies.

In Li et al.'s study performed with fresh samples, the HPV-DNA prevalence was higher than in FFPE samples. Those authors stated that no DNA damage occurred, and that the possibility of HPV-DNA detection might therefore be higher without the formalin fixation steps [16]. Sarıer et al. also used fresh tissues, and observed significantly higher HPV-DNA positivity in the patient group [24]. However, in another study by Llewellyn et al. using the combined assay for HPV16/HPV18 sequences to analyze the DNA extracted from fresh frozen tissue specimens, the authors obtained a positive result for HPV in one out of the 689 UBCs tested (0.1%) whereas the internal control was positive in all cases [25]. Different results can thus be obtained with fresh tissues. In the light of these different findings, there is still no generally accepted method that provides a high rate of nucleic acid in fresh or FFPE tissues.

In conclusion, the role of HPV infection in the etiology of bladder cancer is still unclear, and the impact on bladder cancer development continues to be controversial. However, the majority of studies have reported a relationship between bladder cancer and HPV. However, the rate of detection of HPV, which plays an active role in the etiology of cervical cancer, in bladder cancer was 3.3% in this study. The differences in detection rates may be related to HPV prevalence in the populations in which studies are conducted. Further studies

involving large and different populations are needed to confirm the association of bladder cancer with HPV reported in previous studies. In addition, the importance of carefully monitoring patients diagnosed with HPV type 16 and 18 infections and informing patients in terms of risks is self-evident.

Scientific Responsibility Statement
The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: *This study was supported by research funding from Aydin Adnan Menderes University with ADU-BAP-TPF-12014 fund number.*

Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

References
1. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013; 49(6): 1374.
2. Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. Eur Urol. 2013;63(2):234-41.
3. Boyle P. International Agency for Research on Cancer, World Health Organization. World cancer report 2008. Lyon: IARC Press; 2008.
4. Wood PD. Tumors of the Bladder. In: Wein AJ, Navousi LR, Partin WA, Peters CA, editors. Campbell Urology. 11th ed. Philadelphia, PA: Elsevier; 2016. p:2184-205
5. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. Lancet Glob Health. 2016; 4(9):e609–16.
6. Kjaer S K, Frederiksen K, Munk C, Iftner T. Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. J. Natl Cancer Inst. 2010; 102(19): 1478–88.
7. Ben Selma W, Ziadi S, Ben Gacem R, Amara K, Ksiaa F, Hachana M, et al. Investigation of human papillomavirus in bladder cancer in a series of Tunisian patients. Pathol Res Pract. 2010;206(11):740-3
8. Münger K, Baldwin A, Edwards EM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papilloma virus induced oncogenesis. J Virol. 2004;78(21):11451–60.
9. Kitamura T, Yogo Y, Ueki T, Murakami S, Aso Y. Presence of human papilloma virus type 16 genome in bladder carcinoma in situ of a patient with mild immunodeficiency. Cancer Res. 1988;48:7207-11.
10. Anwar K, Naiki H, Nakakuki K, Inuzuka M. High frequency of human papilloma virus infection in carcinoma of the urinary bladder. Cancer. 1992; 70(7): 1967-73
11. Barghi MZ, Hajimohammedmehdiarbab A, Moghaddam SM, Kazemi B. Correlation between human papillomavirus and bladder transitional cell carcinoma. BMC Infect Dis. 2005; 5: 102-6.
12. Shigehera K, Sasagawa T, Kawaguchi S. Etiolojic role of human papilloma virus infection in bladder carcinoma. Cancer. 2011; 117(10): 2067-76.
13. Berrada N, Al-Bouzidi A, Ameur A. Human papillomavirus detection in Moroccan patients with bladder cancer. J Infect Dev Ctries. 2013; 7: 586-92.
14. Westenend PJ, Stoop JA, Hendriks JG. Human papilloma viruses 6/11, 16/18 and 31/33/51 are not associated with squamous cell carcinoma of the urinary bladder. BJU Int. 2001;88(3): 198-201.
15. LaRue H, Simoneau M, Fradet Y. Human papilloma virus in transitional cell carcinoma of the urinary bladder. Clin Cancer Res.1995;1(4):435-40.
16. Li N, Yang L, Zhang Y, Zhao P, Zheng T, Dai M. Human papillomavirus infection and bladder cancer risk: a meta-analysis. J Infect Dis 2011; 204(2):217–23.
17. Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, et al. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when? PLoS One. 2007;2(6):e537.
18. Daugaard I, Kjeldsen TE, Hager H, Hansen LL, Wojdacz TK. The influence of DNA degradation in formalin-fixed, paraffin-embedded (FFPE) tissue on locus-specific methylation assessment by MS-HRM. Exp Mol Pathol. 2015;99(3):632-40.
19. Auerbach C, Moutschen-Dahmen M, Moutschen J. Genetic and cytogenetical effects of formaldehyde and related compounds. Mutation Research/Reviews in Genetic Toxicology. 1997; 39(3-4):317–61.
20. Von Ahlfen S, Missel A, Bendrat K, Schlumpberger M. Determinants of RNA quality from FFPE samples. PLoS One. 2007; 2 :12.
21. Do H, Dobrovic A. Sequence Artifacts in DNA from Formalin-Fixed Tissues: Causes and Strategies for Minimization. Clin Chem. 2015;61(1):64 LP-71.
22. Hedegaard J, Thorsen K, Lund MK, Hein AMK, Hamilton-Dutoit SJ, Vang S, et al. Next-Generation Sequencing of RNA and DNA Isolated from Paired Fresh-Frozen and Formalin-Fixed Paraffin-Embedded Samples of Human Cancer and Normal Tissue. PLoS One. 2014;9(5):e98187.
23. Robbe P, Popitsch N, Knight SJL, Antoniou P, Becq J, He M, et al. Clinical whole-genome sequencing from routine formalinfixed, paraffin-embedded specimens: pilot study for the 100,000 Genomes Project. Genet Med. 2018;20(10):1196-1205.
24. Sarier M, Sepin N, Keles Y, Imir L, Emek M, Demir M, et al. Is There any Association between Urothelial Carcinoma of the Bladder and Human Papillomavirus? A Case-Control Study. Urol Int. 2020;104(1-2):81-6.
25. Llewellyn MA, Gordon NS, Abbotts B, James ND, Zeegers MP, Cheng KK, et al . Defining the frequency of human papillomavirus and polyomavirus infection in urothelial bladder tumours. Sci Rep. 2018;8(1):11290.

How to cite this article:
Mehmet Yıldızhan , İzzet Koçak, Sevin Kırdar, Nil Çulhacı. Detection of Human Papillomavirus using polymerase chain reaction methods in transitional urothelial bladder cancer. Ann Clin Anal Med 2021;12(4):353-357